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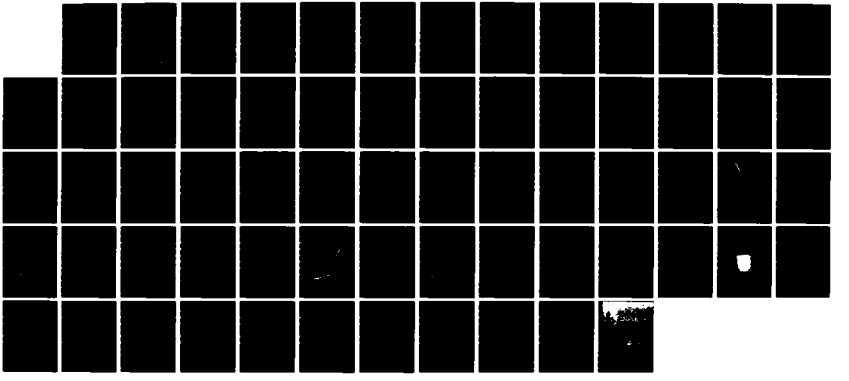

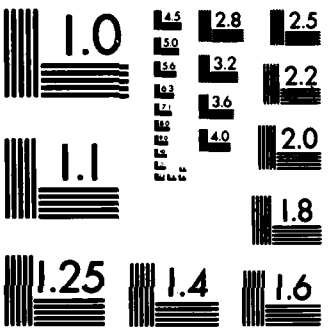


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# THE ROLE OF recA PROTEIN IN THE MULTIPLICITY REACTIVATION PATHWAY OF PHAGE T4

by

Ronald Patrick McCreary

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A Thesis Submitted to the Faculty of the

COMMITTEE ON GENETICS (GRADUATE)

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN GENETICS

In the Graduate College

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1983

## STATEMENT BY AUTHOR

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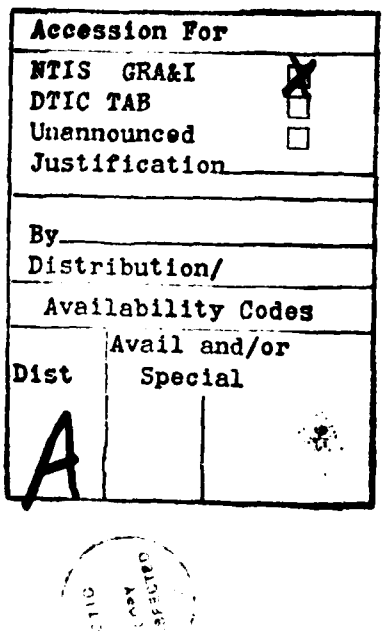
To Jane and Brian

they make it all worthwhile



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# INTRODUCTION

One of the first DNA repair mechanisms to be identified was described by S. E. Luria (1947). He reported that if T-even bacteriophages (T2, T4, or T6) are irradiated with ultra-violet light and then allowed to infect at a multiplicity of two or more they had a greater survival rate than if the multiplicity of infection was kept at one or less. This type of repair, now known as multiplicity reactivation (MR), is generally interpreted as resulting from the action of a recombinational repair pathway determined by genes mainly coded for by the phage. The evidence supporting this view of MR stems from two main lines of investigation (Bernstein and Wallace, 1983). First, the requirement that the host cell contain at least two phage chromosomes directly implicates a recombinational mechanism. Second, there is considerable genetic evidence showing that several of the genes required for MR are also required for maintaining normal levels of recombination between markers.

Genetic studies of both spontaneous recombination and MR show that at least seven genes that are required for recombination in T4 are also required in the expression of the MR pathway. These genes are 32 (single-stranded DNA

binding protein), 46 and 47 (coding for an exonuclease), 59, uvsW, uvsX, and uvsY (coding for recA like functions) (Bernstein, 1981; Bernstein, 1983). These genes have been reported as functioning not only in the MR of UV damaged phage but also in the MR of nitrous acid and mitomycin C treated phage as well. Additionally, two host genes, polA and recA, both known to be involved in E. coli recombination, have been shown to also be involved in MR (Bernstein, 1981; Primer and Chan, 1978; this work). This evidence strongly implicates a recombination type of mechanism as the primary candidate to explain MR.

## A Pathway of Multiplicity Reactivation

In his description of MR, Luria presented a model to explain the phenomenon that ((with a modification by Rayssiguier et al. (1980))) still fits the current data. Known as the "partial replica" model, it requires that there be muliple origins of replication within the phage chromosome that are "activated" independently of any DNA damage. In cells multiply infected by two or more damaged phage chromosomes, those regions of the DNA not affected would replicate their "units" in excess, creating "spare parts" for phage multiplication. These excess "parts" could either replace damaged areas in other chromosomes or combine to make an intact, undamaged chromosome.

Using this model, Bernstein and Wallace (1983) have proposed a pathway which outlines the major reactions of MR. As shown in Figure 1, the pathway begins with DNA replication carried out by the phage replication enzymes. The replication fork proceeds until coming to an area of DNA damage. The replication machine then leaves a gap in the new strand opposite the lesion. This marks the area where MR will initiate. The second step in this proposed pathway involves the nucleases coded for by genes 46 and 47 which would "strip" the area around this replication gap enlarging the area of single-stranded DNA (ssDNA). At this point the second phage chromosome is brought into the pathway where it undergoes "cutting in-trans" by an undefined set of enzymes. This creates an area of ssDNA in the second chromosome which is complementary to the single strand gap in the first chromosome. The next step is the homologus pairing of the two complementary regions of DNA and heteroduplex joint formation. This is thought to be accomplished by the gene products of uvsX, uvsY, host recA, and possibly uvsW. Essentially, the remainder of the pathway is identical to that in conventional models of recombination (ie. involving undamaged chromosomes). These steps are branch migration, Holliday structure resolution, the completion of repair DNA synthesis, and the ligation of remaining nicks.

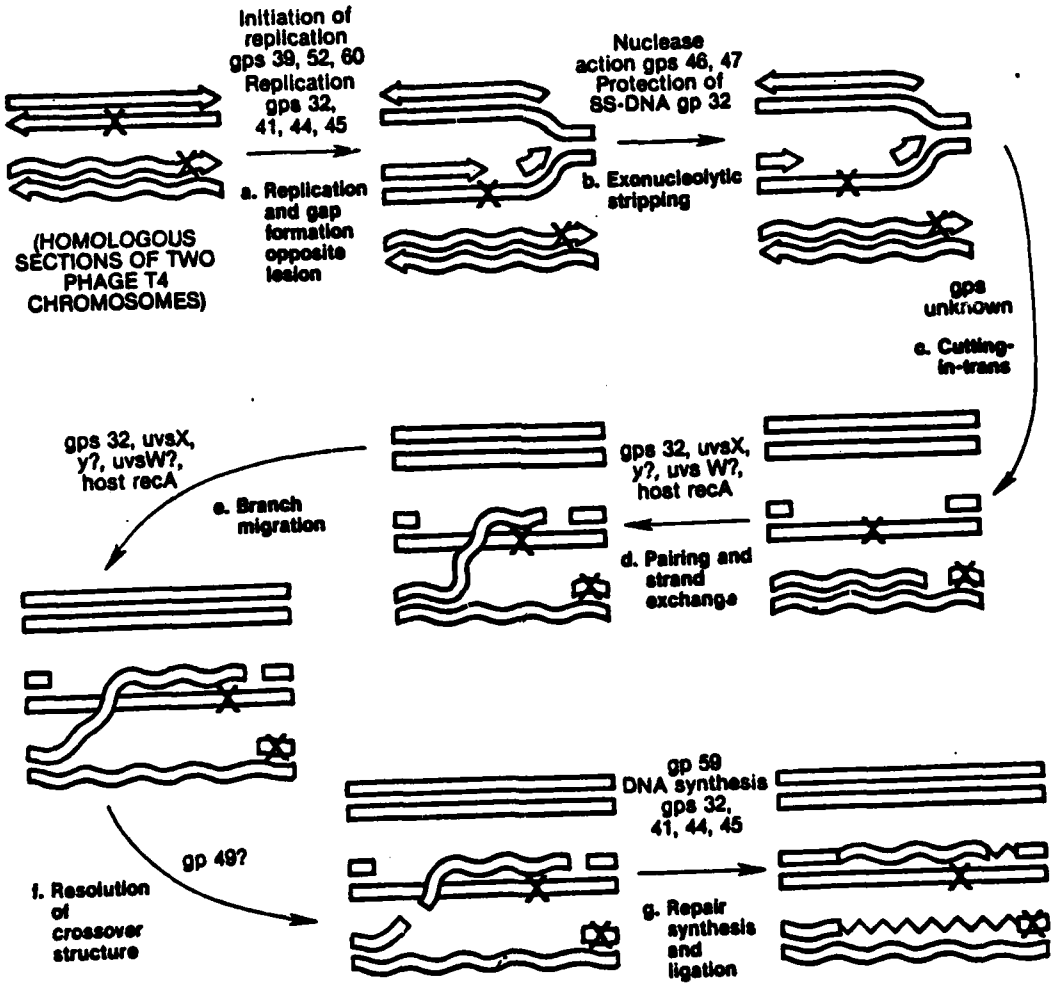


Figure 1. A Model of MR in T4-infected Cells (from Bernstein and Wallace, 1983, used with permission)

The Bernstein-Wallace pathway includes all known gene products that have been directly shown to be required for MR. It also accommodates information on the enzymatic roles of each of the gene products involved. There are several steps where the enzymology is not known or completely understood, such as the resolution of the crossover structure. However, each step is compatible with the current, accepted models of both MR and general recombination.

## Escherichia coli recA Gene Product

The E. coli recA gene was initially identified by Clark and Margulies (1965; also see Clark, 1973) when they demonstrated that a recA mutant had dramatically reduced genetic recombination. Utilizing a surviving fraction assay, Primer and Chan (1978) reported that MR of UV irradiated phage T4 could be demonstrated only in a $recA^+$ host but not in a $recA^-$ one regardless of the genotype of the phage tested. In the last few years this essential recombination protein has been the subject of many investigations and a relatively complete picture of its functions is beginning to be developed.

The recA gene product is a bifunctional protein. First, it has a highly specific protease activity (Craig and Roberts, 1980; Little et al., 1980). Second, it

binds to ssDNA, has a DNA-dependent ATPase activity, and promotes pairing of homologous DNA (Shibata et al., 1979; Weinstock, et al., 1979; Radding, 1981). The monomeric form of the protein has a molecular weight of approximately 38,000 (Radding, 1982). The recA gene has a coding capability for a 353 amino acid polypeptide. Thus far, partial amino acid sequencing of the purified protein has been in agreement with that expected from the DNA sequence. The active form of the enzyme does not seem to be the monomer, however, since the ATPase activity appears to reside in a tetrameric form (Ogawa et al., 1978; Radding, 1981). At neutral pH, in the presence of ATP, the protein tends to form filaments that, under the electron microscope, appear to be a few monomers in width and up to 50 monomers in length (Radding, 1982). Furthermore, in the presence of the non-hydrolyzable ATP analog ATP-$\gamma$-S the protein will form long branched structures (Flory and Radding, 1982; West et al., 1981).

As with the pathway for multiplicity reactivation described above, most models for recombination, including the Holliday model (Holliday, 1964), the Meselson-Radding model (Meselson and Radding, 1975) and the Interwrapping model (Potter and Dressler, 1976; Dressler and Potter, 1982), include a key step where a segment of ssDNA is inserted into the homologous region of a double-stranded helix.

The finding of Weinstock et al. (1979) that recA, in the presence of ATP, promotes the rapid renaturation of complementary single strands of DNA plus the generally accepted requirement for such an activity in recombination led to several intense biochemical studies of the protein.

The recombinational activity of the recA gene product can be divided into three separate functions: 1) pairing of homologous segments of DNA or synapsis, 2) the strand transfer or strand uptake reaction, and 3) the movement of the crossover structure along the double helix or branch migration. The synapsis reaction, like all the identified recA functions, requires ssDNA as a cofactor (Radding, 1981; West et al., 1980; Shibata et al., 1979). The formation of D-loops, the stable structure formed after the synapsis and strand transfer are completed, occurs optimally in vitro when the stoichoimetry of recA to ssDNA is held to a ratio of 1 molecule per 3-5 nucleotides (McEntee et al., 1981). This is also the calculated binding ratio of recA to ssDNA. The kinetics of the recA catalyzed synapsis follows classical enzyme kinetics indicating that the rate limiting step is a ternary complex. This complex likely consists of recA, ssDNA, and double-stranded DNA (dsDNA) forming a relatively unstable pre-D-loop structure (Radding et al., 1980; Cassuto et al., 1980). The existence of this intermediate structure was also

inferred by Shibata et al. (1979) and McEntee et al. (1980). They reported that when synapsis is carried out in the presence of ATP-$\delta$-S, a filter binding intermediate consisting of DNA and protein is formed that could be dissociated by protein denaturing agents. These results implied a mechanism of random initial pairing of the recA to ssDNA at regions of nonhomology forming the ternary complex. Apparently, the search for homology then takes place with the hydrolysis of ATP (Dressler and Potter, 1982).

After synapsis has occurred, strand uptake proceeds in a polar fashion (Kahn et al., 1981). Using a system involving the use of chimeric duplex DNA fragments with M13 sequences at one end and G4 sequences at the other, the authors were able to examine the strand transfer reaction as catalyzed by recA. They found a strong bias (greater than 40:1) favoring the pairing of the 3' end of the ssDNA with the concurrent displacement of the helix occuring at the 5' end. The polar, unidirectional nature of this reaction would be expected in most recombination models to lead to the production of long linear heteroduplex molecules. Such molecules, some in excess of 1,000 base pairs in length, have been observed (Weinstock et al., 1979; Dahn et al., 1981).

To achieve extended heteroduplex joint molecules it is necessary that there be a "growth" mechanism present which the recA protein seems able to accomplish with only its cofactors in the reaction mixture. Cox and Lehman (1981) have reported that recA can promote branch migration at a rate of about four base pairs per second. Livneh and Lehman (1982) have shown that not only does recA promote branch migration of intact DNA but that it can catalyze the complete exchange of strands containing pyrimidine dimers at a rate of about 1/50 normal. In addition, they reported that the bypassed dimers are removed from the DNA by excision repair enzymes. recA protein has also been reported to promote branch migration of homologous strands containing sections of nonhomology of up to 650 base pairs in length.

As the experimental evidence mounts, it is becoming quite clear that the recA protein performs the central step in E. coli recombination of annealing a single-stranded portion of DNA to its complementary strand in an intact double helix. However, this is not the whole story. As mentioned above, recA is a bifunctional protein whose other role is that of a protease, and it is toward that aspect of the molecule that I now turn my attention.

The initial observations that led to the conclusion that recA is a protease were that treatment of E. coli with

DNA damaging agents such as ultraviolet light, or blocking its DNA replication, induces the synthesis of a novel protein now identified as the recA protein (Gudas and Pardee, 1976; Little and Hanawalt, 1977; Emmerson and West, 1977). Radman (1975) and Witkin (1976) proposed that this protein induction was part of a unified cell response, now called the SOS response, to DNA damage or replication arrest. They speculated that the recA protein might somehow be regulating this response.

Two main substrates for the recA protease have been identified. Craig and Roberts (1980) reported that the λ phage repressor protein was cleaved by the protease in the presence of polynucleotides, and Little et al. (1980) reported that the lexA gene product was also cleaved by the activated recA protease. With these observations plus an ever increasing amount of genetic evidence implicating lexA as being involved in the regulation of the SOS response, a model for the control of the repair system was formulated (McPartland et al., 1980).

In describing this model, one can begin with the undamaged replicating cell. Here the lexA gene is producing lexA protein which acts as a repressor for all the other genes in the SOS system including the recA gene. The repression is not complete, however, allowing for a low level of expression of most of the proteins, eg. constitutive

levels of recA seem to be about 2,000 molecules per cell. When the bacterial DNA is damaged or replication is stopped, the recA protease is activated by an undetermined inducing signal and begins cleaving the lexA repressor molecules. As the repressor is eliminated, the SOS pathway becomes "turned on" and full expression of its constituent enzymes occurs. For example, the concentration of recA increases to around 50,000 molecules per cell or approximately 6% of the total cell protein. In cells lysogenic for phage λ , the repressor which maintains the lysogenic state has apparently evolved a sensitivity to the activation of the recA protease as its signal that all is not well with its host, and that it is time to leave. Lysogenic phage λ then initiates its lytic life cycle by having its repressor cleaved by activated recA protease. In non-lysogenic cells, the main function of the SOS repair pathway is to overcome DNA lesions. As lesions are removed, the inducing signal that has kept the protease active begins to dissipate and a concurrent decrease in enzyme activity is seen. The result of this is a gradual build up of the lexA repressor which begins to shut down the system completing the cycle. (McPartland et al., 1980; Gottesman, 1981; Radding, 1982; Little et al., 1982).

When taken as a whole, it is striking that any one protein can have such diverse roles as recA seems to

have. In this one relatively small protein one finds the wherewithal to orchestrate not only the major task of recombination, but also the ability to turn on a network of responses to DNA damage.

## T4 uvsX Gene Product

Radding (1981) addressed the central role of recA in E. coli recombination stating. "...the remarkable economy with which (recA)...accomplishes both of the central steps of recombination...suggests a general mechanism based on a few principles--a mechanism that we can expect to find in operation in many systems of recombination." This expectation is now receiving experimental verification with the finding in different organisms of gene products that are very similar to recA. One example is the recE gene product of Bacillus subtilis (deVos and Venema, 1982). The Proteus mirabilis recA gene product and the large T antigen of SV40 (West et al., 1983; Seif, 1982) have been shown to have anti-recA antibody crossreactivity.

In 1982, Minagawa, in a personal communication to C. Bernstein, stated that the T4 gene uvsX codes for a protein that has many of the same functions as the recA protein (T. Minagawa, Kyoto Univ., Kyoto, Japan, May 1982). He related that while the uvsX gene product is immunologically distinct from recA, it has a similar molecular weight (40,000), has an ssDNA dependent ATPase activity, and

promotes the pairing of homologous DNA. He reported that the T4 ssDNA binding protein, gp32, enhanced the DNA pairing reaction of the uvsX protein in much the same way as the E. coli ssDNA binding protein enhances the recA catalyzed reaction. The interaction of gp32 and gpuvsX has been confirmed by Formosa et al. (1983) with the finding that a gene 32 affinity column retains the uvsX protein.

The uvsX gene of phage T4 was first described by Harm (1964) as a gene involved in the repair of UV damage and genetic recombination. Investigators have, however, obtained conflicting data as to its role in MR. Several authors have reported that this gene is needed for the MR of UV damaged phage (for a review see Bernstein, 1981). However, Primer and Chan (1978) reported that MR of UV irradiated phage did occur when a uvsX mutant was tested. Another area where reported results seem to be contradictory is on the MR of Mitomycin C. (MMC) damaged phage. When MR was measured by a surving fraction assay, the data suggested that the uvsX gene is not required (Homes et al., 1980). However, Shimizu and Sekiguchi (1974), measuring the amount of DNA synthesis occurring, concluded that the uvsX gene is required for MR of MMC damaged phage. Due in part to these conflicting genetic data and the similarity in functions between the products of it and recA, the uvsX gene of T4 and the protein it encodes are becoming the focus of experimentation in several laboratories.

## Mitomycin C

Mitomycin C is a well characterized DNA damaging agent that has been utilized in cancer chemotherapy. It has been shown to be mutagenic in E. coli and to induce many, if not all, recA dependent functions at high concentrations (Iifima and Hagiwara, 1960; Lossius et al., 1983; Barbe et al., 1983). Holmes et al. (1980) reported an increase in recombination between genetic markers of T4 after exposure to relatively low concentrations of the drug.

Studies done by Iyer and Szybalske (1963) and Tomasz et al. (1974) indicate that, at low doses, MMC induces approximately 200 lesions per E. coli chromosome of which about 10% were cross-links and 90% were monadducts. Its specific mode of action appears to be to first intercalate into the helix in a noncovalent manner and then to covalently bond to the DNA, most likely with guanine (Tomaxz et al., 1974).

## Statement of the Problem

Multiplicity reactivation is a recombinational repair pathway of phage T4 that can overcome lethal lesions in the phage chromosome. The pathway is catalyzed by the products of several phage genes and at least two host genes that are associated with replication, recombination, and other repair pathways (Bernstein, 1981; Bernstein and Wallace, (1983). Further information on this repair

pathway and on the nature of the interaction between the phage and the host gene products should help improve our understanding of the mechanism and significance of recombinational repair pathways.

The purpose of this study was to obtain further information on the roles of the host recA gene and the phage uvsX gene in the MR of mitomycin C induced lesions. In an effort to accomplish this, the MR of mitomycin C treated phage/host complexes was studied utilizing two recA mutants, a uvsX mutant, and by varying the experimental conditions.

## MATERIALS AND METHODS

The bacterial and phage strains used in this work are listed in Table 1. The Escherichia coli strains AT713, GY3428, and GY3448 were obtained from Dr. Barbara Bachman, E. coli Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut, 06510. E. coli strain S/6/5 and both phage strains were obtained from the stock collection maintained by Dr. Harris Bernstein, Department of Molecular and Medical Microbiology, University of Arizona College of Medicine, Tucson, Arizona, 85724.

### Media

The following growth media were used:

1. Hershey broth (Steinberg and Edgar, 1962)

| Component | Amount (g/L) |
|---|---|
| Bacto Nutrient Broth (Difco) | 8.0 |
| Bacto Peptone (Difco) | 5.0 |
| NaCl | 5.0 |
| Glucose | 1.0 |
| pH adjusted to 7.2-7.4 with 5N NaOH | |

Table 1. Bacterial and Phage Strains

| Strain | Relevant Genotype | Repair Phenotype | Reference |
|---|---|---|---|
| *E. coli* | | | |
| AT713 | Wild Type | Wild Type | Taylor and Trotter (1967) |
| GY3428 | recA431 | Protease$^+$ Recombination$^-$ | Morand, et al. (1977) |
| GY3448 | recA430 | Protease$^-$ Recombination$^+$ | Morand, et al. (1977) |
| Bacteriophages | | | |
| T4D | Wild Type | Wild | |
| px | uvsX$^-$ | Recombination$^-$ | Harm (1964) |

2. M9 complete medium (Adams, 1959)

| | |
|---|---|
| $NaHPO_4$ | 6.0 |
| $KH_2PO_4$ | 3.0 |
| $NH_4Cl$ | 1.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.25 |
| Glucose | 40.0 |
| $MgSO_4$ | 0.27 |
| $FeCl_3$ | 2.7 |
| Case amino Acids--Vitamin Free (Difco) | 2.5 |

Ph Adjusted to 6.8 with 0.2N HCL

Both media were supplemented with 40.0mg/L of thymine when used to grow strains AT713, GY3428, and GY3448.

Plating agar was made as described by Steinberg and Edgar (1962):

| | g/Liter |
|---|---|
| Bacto agar (Difco) | 10.0 |
| Bacto Tryptone (Difco) | 13.0 |
| NaCl | 8.0 |
| Sodium Citrate | 2.0 |
| Glucose | 3.0 |

Soft agar (top agar) was made with the same ingredients as plating agar except the agar concentration was lowered to 6.5g/L and the glucose reduced to 1.3g/L (Steinberg and Edgar, 1962).

Absorption salts (M9 salts) contained $NaHPO_4$, $KH_2PO_4$, $NH_4Cl$, and $MgSO_4 \cdot 7H_2O$ at the same concentrations used in M9 complete (Adams, 1959).

## Phage Stock Preparation

High titer phage stocks were prepared using E. coli S/6/5 as the host. The cells were grown to about $5x10^7$ cells/ml in 250ml of Hershey broth at 37°C with aeration. Approximately $10^5$-$10^6$ phage was then added and allowed to grow until cell lysis was visable (usually a minimum of 5 hrs.) or overnight. A few drops of $CHCl_3$ were added to lyse any remaining cells and the cell debris was removed by centrifugation in a Sorvel GSA roter (3,000 rpm for 15-20 min.). The supernatant was divided into six equal portions and the phage pelleted using a Sorvel SS34 rotor (16,000 rpm for 2 hrs.). The supernatant was discarded and the pellets gently resuspended in 2ml of M9 salts. The portions were combined and the phage concentration assayed.

All phage and bacterial assays were conducted using the soft agar overlay technique described by Adams (1959). A dilution of bacteria or phage plus three to five drops of concentrated indicator bacteria was added to approximately 3ml of melted top agar and the mixture poured onto an agar plate in an even layer. Indicator bacteria were prepared by growing 250ml of strain S/6/5 to $3-4x10^8$ cells/ml in Hershey broth, pelleting them at 3,000rpm for 15-20 minutes,

and resuspending them in 15-20ml of fresh broth. In all tests, the plates were incubated at 37°C for 18-24 hours and the phage plaques were counted using a New Brunswick Scientific counter.

The phage stock was stored at 4°C over 2-3 drops $CHCL_3$. A fresh stock was prepared when the titer dropped below $5x10^{10}$ phage/ml.

Repurification of uvsX by Cross to Wild Type

Repurification of the uvsX T4 mutant was performed essentially as described by Fisher and Bernstein (1969). The cells were grown to about $2x10^8$ cells/ml in Hershey broth with aeration, washed twice with M9 salts, and starved for one hour at 37°C with aeration. A 10ml aliquot was taken and simultaneously infected with both T4D and $p_x$ at a multiplicity of infection, MOI, of five each (total MOI of 10). The phage were allowed 15 minutes for absorption at 37°C without aeration. The infected cells were then diluted and plated using the soft agar overlay technique previously described. Ten plaques were selected and each aspirated with a Pasture pipet into 1.5ml of Hershey's with 2 drops of $CHCl_3$. The UV sensitivity of each of these phage clones was determined by irradiating a 0.8ml sample with a GE G8T5 bulb at 22.5cm yielding a measured energy of 20 ergs/$mm^2$/sec. The samples were irradiated for periods of 5, 10, 20, 30, and 40 seconds with the surviving fraction measured at each

point by soft agar overlay. The most UV sensitive clone was checked for absorption and a high titer stock was prepared.

## T4 Antiserum Preparation

Two female New Zealand white rabbits were used to produce antiserum to phage T4. One mililiter of T4D stock (approximately $10^{11}$ total phage) was mixed with an equal volume of Freund's Complete Adjuvant, and each rabbit was given 1ml of this mixture in six equal intradermal injections along their backs. After three weeks a 15 ml blood sample was drawn from each rabbit by ear venipuncture, and a titer of the antibody determined essentially as described in the Cold Spring Harbor Laboratory Manual on Bacterial Viruses (1967). The serum was separated from the plasma by centrifugation and diluted to 1:10, 1:100, and 1:1000. An equal volume of each dilution was added to a known concentration of phage and viable phage assayed at various times following inoculation. The activity was calculated from the equation:

$$P=P_o e^{-(\frac{Kt}{D})}$$ where---P=initial phage concentration

$P_o$=viable phage at time t

K=activity of antisera

D=dilution factor

Following the test bleeding, the animals were given a booster injection identical to the first that was followed five days later with a second injection consisting of 0.4ml

of undiluted phage stock given intermuscularly into a thigh. Ten days after this last injection, another test bleeding was performed which was followed two days later with a final blood collection of approximately 50ml from each animal.

The serum was separated into 1ml aliquots for storage at -70°C until used. Crossreactivity of the antiserum with E. coli was checked at this time and found to be nonexistent.

## Multiplicity Reactivation of Mitomycin C Treated T4

Multiplicity reactivation (MR) experiments were conducted using a modification of the procedure described by Holmes et al. (1980). The cells were grown in either Hershey broth or M9 complete media as indicated to a concentration of approximately $2x10^8$ cells/ml, at 37°C with aeration. The cell concentration was followed visually using a Petroff-Houser counting chamber.

When the cells reached the desired density they were either subjected to a period of starvation prior to infection or were utilized immediately. Starved cells were first washed twice with M9 salts solution and then resuspended in this media where they remained for at least one hour at 37°C with aeration. In those experiments where this starvation step was eliminated, the cells were washed only once with the M9 salts solution, resuspended in this solution and infected immediately.

When the cells were ready for infection, either with or without the starvation step, a viable cell count was performed using the soft agar overlay technique. A 10ml aliquot was then taken and KCN added to a final concentration of 0.001M. Phage were then added at an estimated MOI of $10^{-3}$ for singly infected cells (monocomplexes); or an MOI of approximately 8 for multiply infected cells (multicomplexes). The phage were titered during each experiment and the actual MOI calculated from this titer and the viable cell count.

The phage were allowed 10 minutes for absorption at 37°C after which unabsorbed phage were titered. This free phage assay was done by taking the first dilution into Hershey broth instead of the usual M9 salts solution used for all other dilutions, then lysing the cells with 3-4 drops of $CHCl_3$, and completing the dilutions and plating as described. As rapidly as possible, usually within one minute after absorption was complete, T4 antisera was added at a concentration that would inactivate 99.99% of the free phage in ten minutes at room temperature. During this period the cells were transferred to a 16x125mm capped test tube and placed in an opaque sleeve where they remained for the remainder of the procedure.

Following free phage inactivation, both remaining free phage and total infective centers were assayed in triplicate to establish the initial internal infective center titer just prior to mitomycin C (MMC) treatment.

The MMC (Sigma) was obtained in 2mg vials to which 4.0ml of sterile water was added to yield a working stock at a concentration of $5x10^{-4}$g/ml. This solution was kept in the dark at $4^{o}$C until used, and fresh stock prepared when its potency decreased by 50%. The MMC was added to the phage/host complexes, under yellow light, to a final concentration of $5x10^{-6}$g/ml. At designated time intervals, an aliquot was taken and surviving phage assayed. All dilutions were carried out in parallel, and duplicate plates made for each end point.

To correct for the variability of the MMC activity from day to day, all MR survival curves were normalized against the killing effect on wild type phage monocomplexes for that day. The action of MMC has been shown to follow single hit kinetics (Holmes et al., 1980) and therefore can be described by the equation: $S/S_o=e^{-kd}$ where $S/S_o$=surviving fraction, k=lethal hits, and d=dose. All mitomycin C doses for multicomplexes were normalized against the exposure that resulted in a k=1 ($S/S_o$=37%) for the wild type monocomplexes.

Percent survival for each time point was calculated from the formula:

$$\%\text{survival}=\frac{\text{infective centers}_t-\text{free phage}_o}{\text{internal infective centers}_o} \times 100.$$

## recA Assay

The procedure used to qualitate recA levels in E. coli was adapted from Little and Hanawalt (1977). Cells were grown in M9 complete media to a concentration of 2-3x$10^8$ cells/ml as measured against McFarland standards (MacFaddin, 1980). The cells were processed in 20ml aliquots as described below after UV irradiation and/or starvation for 30 and 60 minutes in M9 salts solution.

Each aliquot of cells was pelleted in a Sorvel SS34 rotor (8,000 rpm for 10 min.) and resuspended in 5ml of Tris-HCl (pH 7.4). The cells were then lysed in an Ultrasonics Inc. Model W225R sonicator (3-five second bursts at 100% duty cycle with 30 sec. cooling in between) and the cell debris pelleted in an SS34 rotor (10,000 rpm for 10 min.). The membrane fraction was then pelleted using a 60Ti rotor at 38,000 rpm for 1 hour in a Beckman L2-65B ultracentrifuge. The pellet was resuspended in 0.5ml Tris-HCl (pH7.4) and frozen until processed for polyacrylamide gel electrophoresis (SDS-PAGE).

The slab gel electrophoresis was performed using a modification of the method described by Laemmli and Fayre (1973). The buffer system and gel composition was as follows (all percentage solutions are w/v unless otherwise indicated):

Electrode buffer

| | |
|---|---|
| Tris-HCL | 0.025M |
| Glycine | 0.19M |
| 10% SDS | 5ml/500ml of buffer |

Sample buffer (4X)

| | |
|---|---|
| Tris-HCL | 1.48ml of a 1M solution, pH 7.8 |
| SDS | 0.8g |
| Glycerol | 8.0ml of a 50% (v/v) solution |
| Bromphenol Blue | 0.4ml of a 0.1% solution |
| Dithiothreotol | 0.14ml of a 1M solution |

Stacking gel

| | |
|---|---|
| Tris-HCL | 1.24ml of a 1M solution pH 6.8 |
| SDS | 1.0ml of a 1% solution |
| Acrylamide-bis | 1.2ml of a 29.2% acrylamide/ 0.8% bis-acrylamide solution |
| Distilled water | 6.28ml |
| Ammonium persulfate | 0.25ml of a 1% solution |
| TEMED | 0.030ml |

Separation gel

| | |
|---|---|
| Tris-HCL | 5.4ml of a 1M solution pH 8.8 |
| Acrylamide-bis | 6.7ml of the 30% solution used above |
| SDS | 2.0ml of a 1% solution |
| Glycerol | 4.0ml of a 50% (v/v) solution |
| Distilled water | 1.4ml |
| Ammonium persulfate | 0.5ml of a 1% solution |
| TEMED | 0.048ml |

Stock solutions were maintained for all of the above except the ammonium persulfate which was prepared just prior to use.

The gels were 7mm in thickness and 11.0cm in total width. The separation gel was poured first to a length of 13.5cm and the stacking gel was poured to a depth of 3cm with a ten well comb inserted. The edges of the gel were covered with Scotch 3M Electrical tape which eliminated any problem with leakage.

The samples were thawed and the optical density of each was read at 280nm on a Gilford 240 spectrophotometer. The same optical density was loaded onto the gel for each sample. The samples were placed in 16x5mm tubes and an appropriate amount of sample buffer was added. In all cases the total volume loaded per sample did not exceed 0.075ml. The samples were then boiled for about one minute and underlayered into the wells. The gel was allowed to run at a constant voltage of 200V until the marker reached the end of the gel. Molecular weight markers were run with each experimental gel.

When the gel was completed, usually after 2-2.5 hours, the plates were carefully separated with the gel sticking to one of them. The stacking gel was removed with a spatula. The running gel was removed with a piece of Whatman #3 filter paper. By pressing the paper firmly onto the gel it could be lifted from the plate and transferred generally without difficulty. The gel was put into a staining basin and fixative added which released it from the paper.

Prior to being photographed, the gel was silver stained in the following manner. Initially it was soaked in fixative ((50% methanol (v/v), 12% acetic acid (v/v)) for 30 minutes to overnight. It was then washed three times with an ethanol/acetic acid solution ((10% ethanol (v/v), 5% acetic acid (v/v))) and submerged in a $K_2Cr_2O_7$-$HNO_3$ solution for 5 minutes ((0.2g $K_2Cr_2O_7$, 0.042ml concentrated $HNO_3$ per 200ml water)). The gel was then washed three times with water and stained for 30 minutes with 0.12M $AgNO_3$. This was followed by two rinses with water. The gel was then developed with a $Na_2CO_3$ solution ((29.68g $Na_2CO_3$plus 0.5ml Formalin per liter)). The developing was stopped by one rinse with distilled water followed by a 1% acetic acid solution (v/v). The gels were then photographed and analyzed.

## RESULTS

Figure 2 depicts the UV sensitivities of five of the clones resulting from the repurification of the uvsX mutant, $p_x$. As shown, the most sensitive clone was number five indicating the most complete mutation of the uvsX gene. A high titer stock was prepared from this clone. The UV sensitivity of wild type T4D is given as a reference.

### MR With Starvation Before Infection

Multiplicity reactivation experiments were carried out with wild type and $p_x$ mutant phage in both wild type and recA$^-$ E. coli. A representative result from each of these experiments is shown in figures 3-6. The MR obtained when both phage and bacteria were wild type is given in Figure 3. The increase in survival of the multicomplexes compared to the monocomplexes, an approximate 12 fold increase, represents the maximum reactivation obtainable under the experimental conditions. A comparison between the results in Figure 3 and those obtained with a recA431 mutant E.coli, one that is recombination deficient and protease proficient, is shown in Figure 4. As can be seen, there is a substantial depression of the MR in the recA431

host as compared to the wild type host (shown by the dashed line). This result is taken to imply that the recombination reactions catalyzed by recA are required for the MR of MMC treated phage. A similar decrease in MR was found when a recA430 mutant E. coli was used as the host. This mutant, deficient in the protease function of recA but not the recombination function, also gave a 2/3 decrease in reactivation (Fig. 5; the wild type reference provided by the dashed line). This result is taken to mean that the protease function of the recA protein is also involved in the MR pathway of MMC treated T4.

The results of MR experiments using the uvsX mutant phage, $p_x$, in a wild type host is given in Figure 6. There was no significant difference in the survival rates of the mutant from that of the wild type, indicating that the uvsX gene product is not required for the full expression of the MR pathway in MMC treated phage.

Figure 7 depicts the results obtained when wild type E. coli was treated with sufficient UV radiation to induce the recA protein (Little and Hanawalt, 1977; Salles and Paoletti, 1983) before infection with wild type phage. There was no significant difference between the MR obtained from the cells that were treated and those that were not (shown by the dashed line).

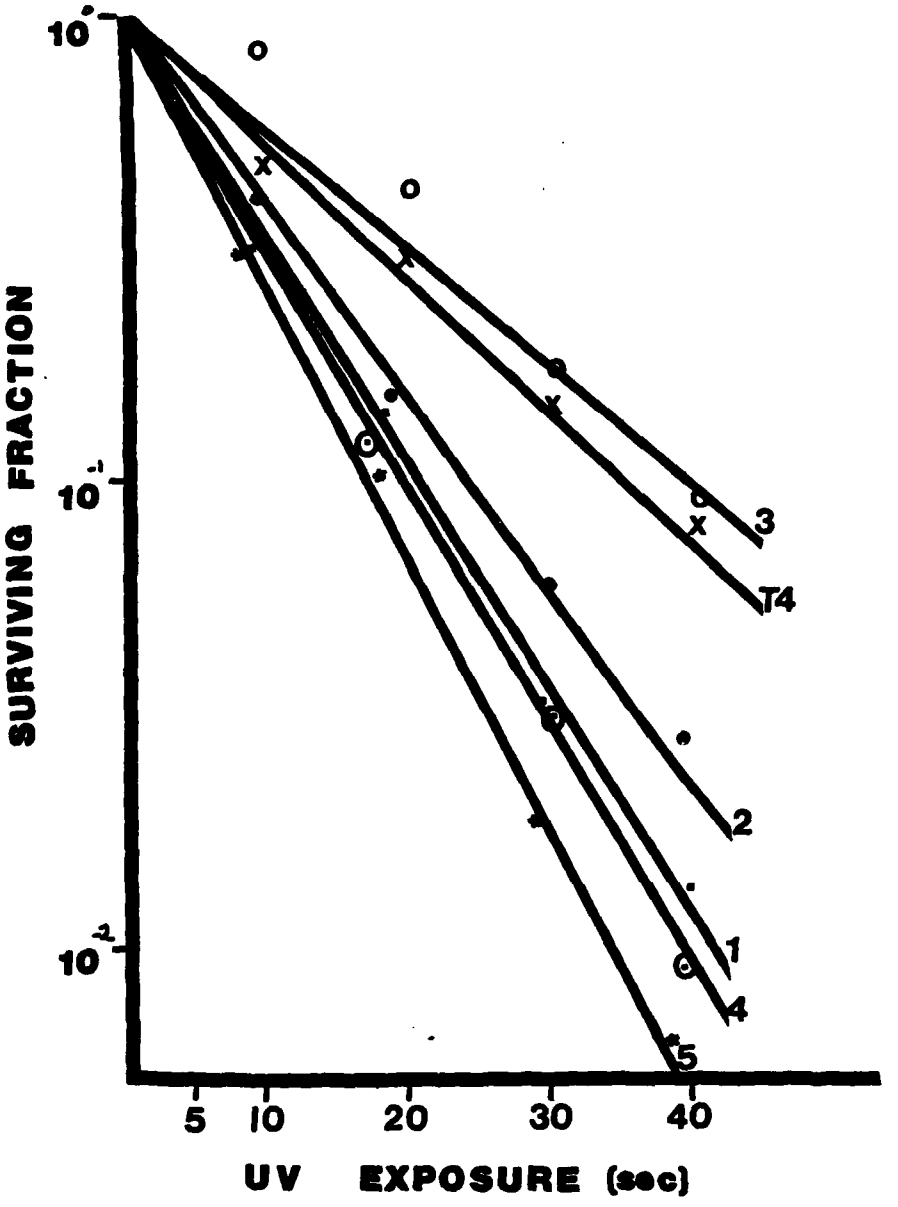


Figure 2. UV Sensitivities of $p_x$ clones following repurification.

× ---T4D
■ ---1
● ---2
○ ---3
⊙ ---4
✱ ---5

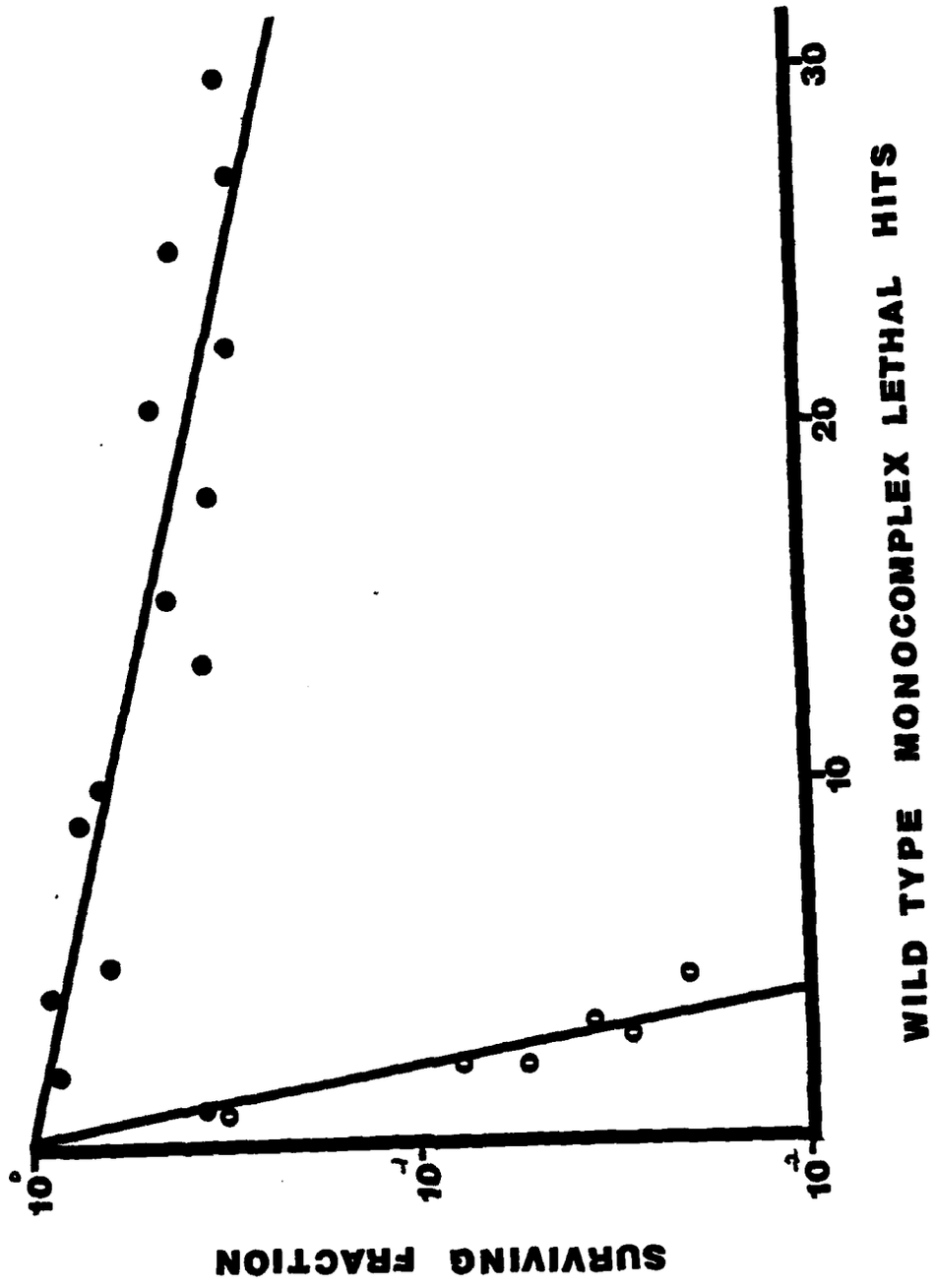


Figure 3. MR with wild type E. coli and T4

● ---Multicomplexes
o ---Monocomplexes

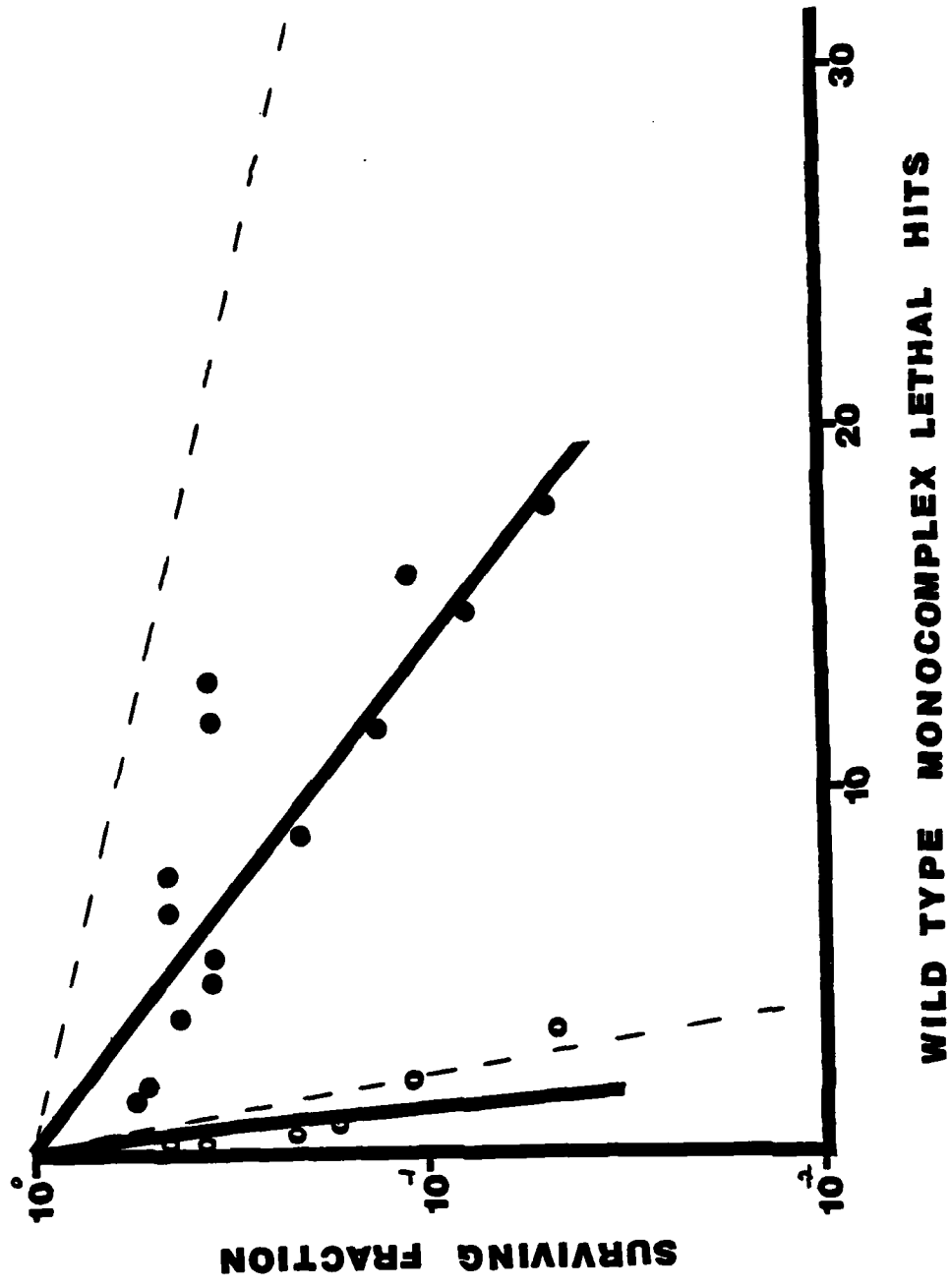


Figure 4. MR with recombination deficient recA⁻ E. coli and wild type T4,

●---Multicomplexes
o---Monocomplexes

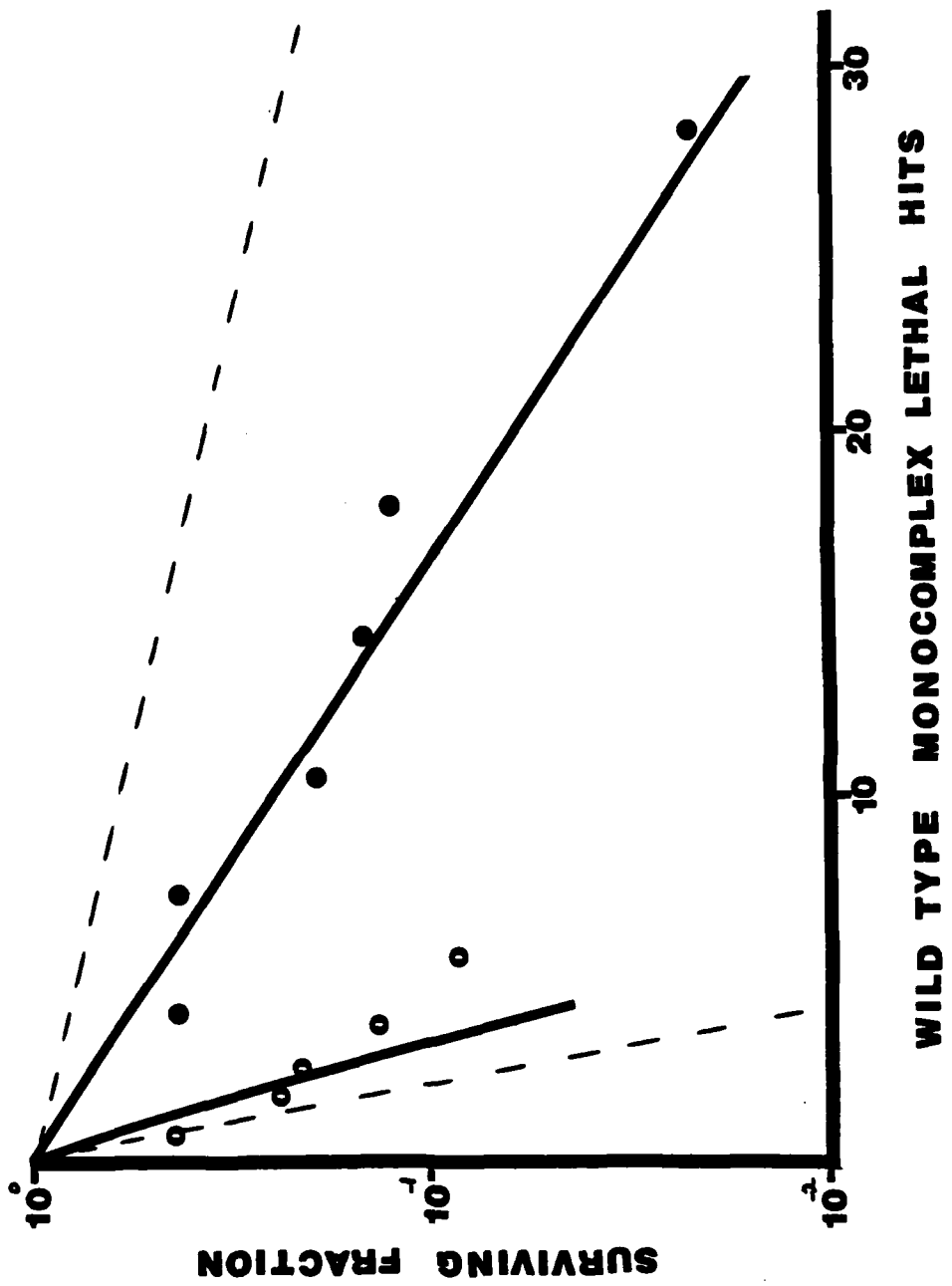


Figure 5. MR with protease deficient recA$^-$ E. coli and wild type T4.

●---Multicomplexes
o---Monocomplexes

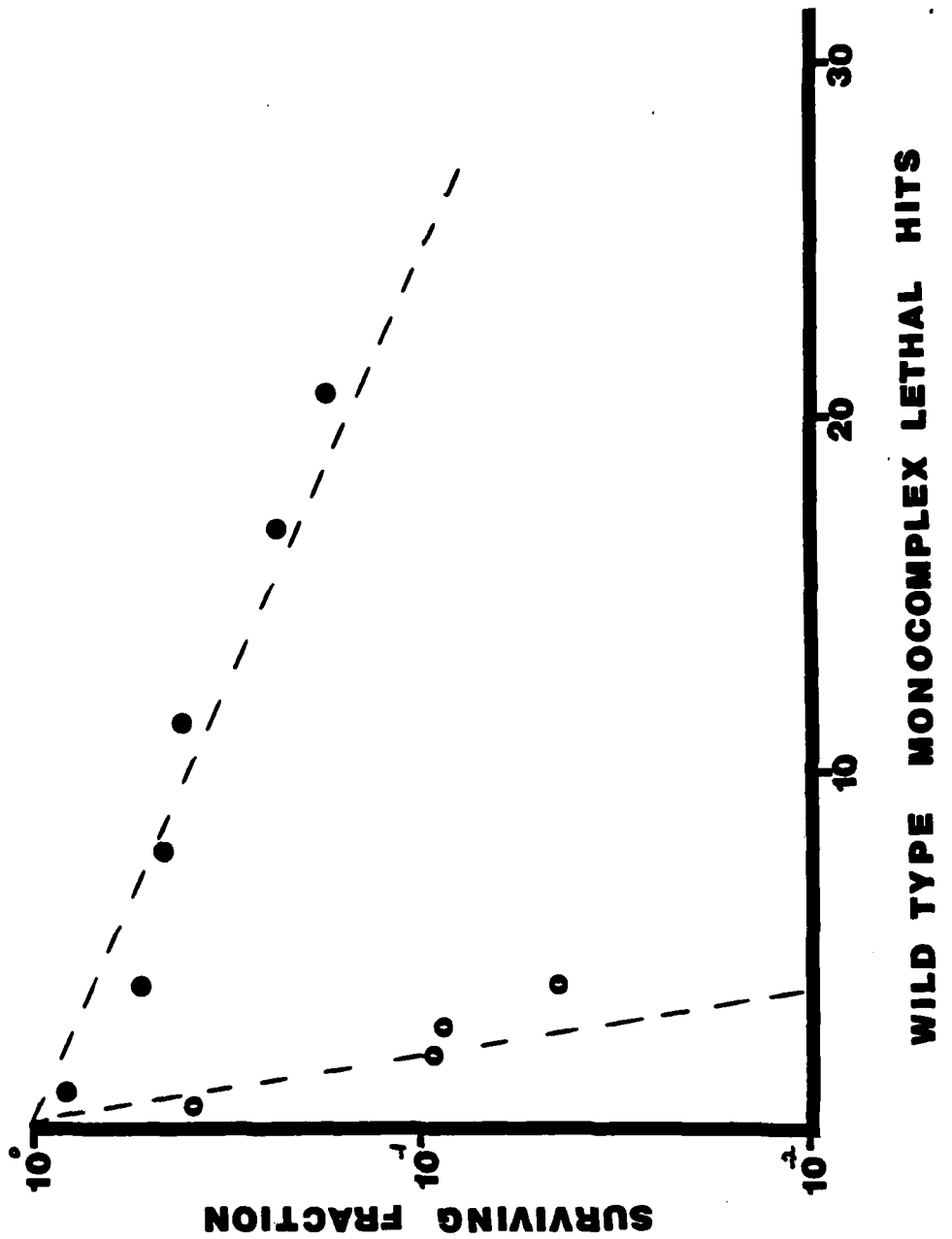


Figure 6. MR with wild type E. coli and recombination deficient uvsX⁻ phage.

●---Multicomplexes
o---Monocomplexes

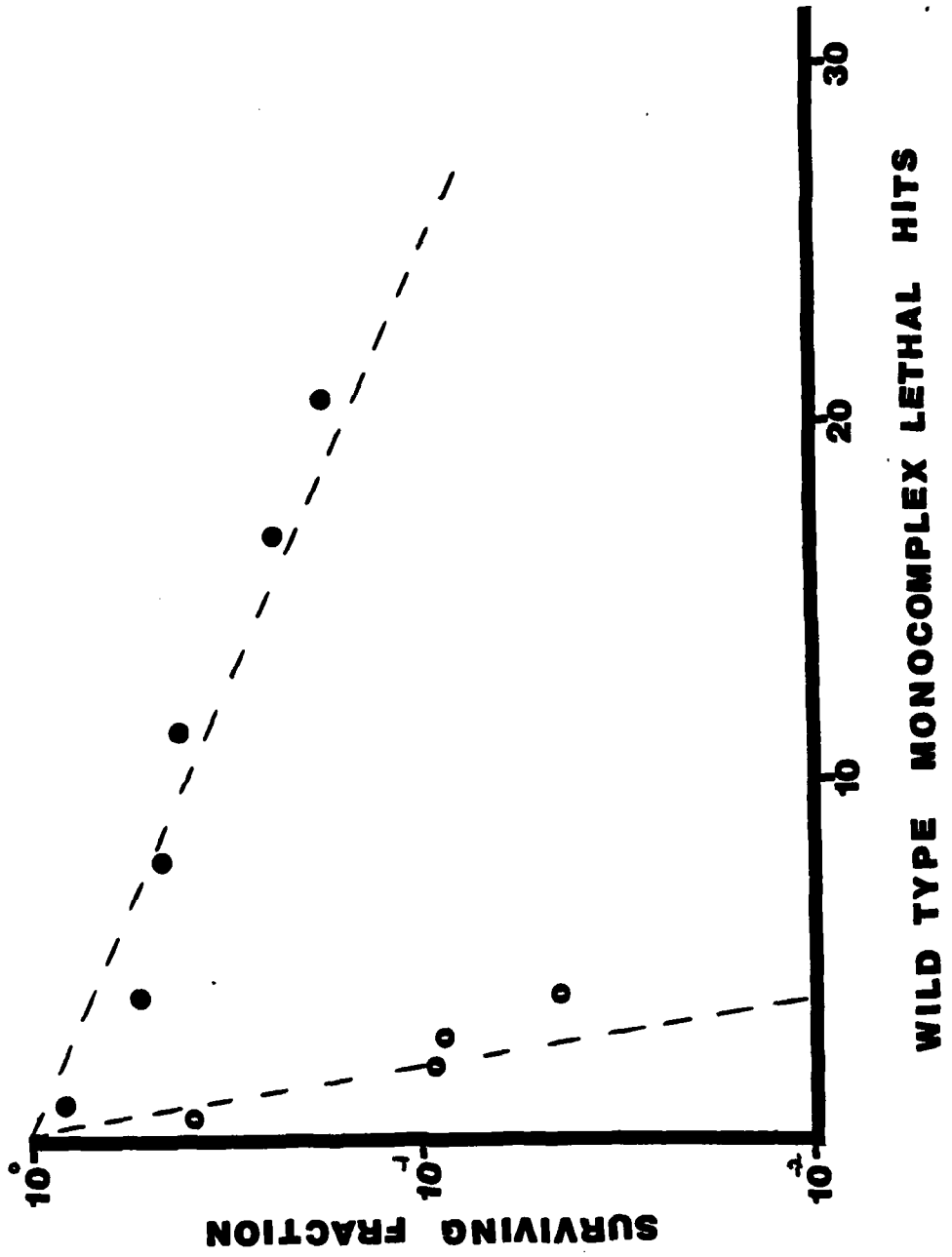


Figure 6. MR with wild type E. coli and recombination deficient uvsX⁻ phage.

●---Multicomplexes
o---Monocomplexes

## Effects of Starvation on Monocomplexes

The effect of four variations to the starvation protocol on monocomplex survival is given in Figure 8. Line 1 shows the results of the starvation protocol without modification. Treatment 1, line 2, involved conducting the absorption step at room temperature instead of at 37°C. Treatment 2, line 3, modified the protocol by adding the MMC five minutes after the start of absorption and before the start of the antisera treatment. In treatment 3, line 4, the cells were chilled on ice from the end of the starvation period through the phage absorption period. Treatment 4, line 5, is the results obtained when the starvation step was omitted entirely from the procedure. Treatments 1-3 did not change the biphasic nature of the monocomplex curves, indicating the presence of a second experimental population, that had been observed in MR experiments performed using the one hour starvation step prior to phage infection. This second population was thought to be the result of some degree of phage replication before MMC treatment. However, a near linear killing curve was obtained, indicating only one population of singly infected cells, only when this starvation step was eliminated from the protocol. From these experiments it was concluded that a DNA repair mechanism nearly as efficient as MR, was inducible following periods of starvation.

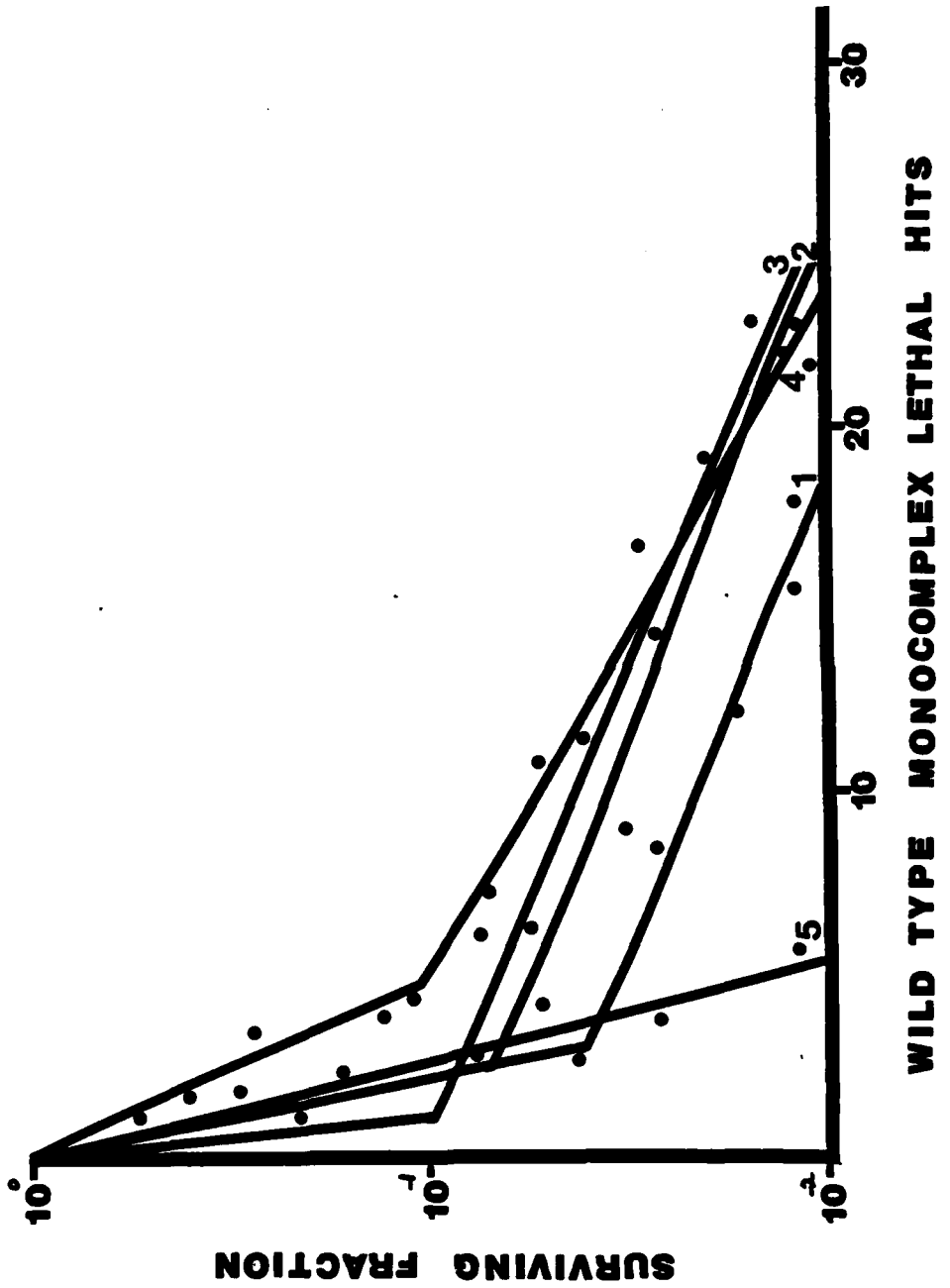


Figure 8. Effects of modifications to the MR protocol.

## Effects of Starvation on MR

The effects of starvation on MR were examined and the results given in Figures 9-11. A summary of the results of all MR experiments is provided in Table 2. Figure 9 shows that there is a decrease in reactivation if the cells are not subjected to the starvation period prior to infection with phage when compared to the same experiment using the starvation step (dashed line).

The effect of eliminating the starvation step was also examined in an recA431 mutant E. coli with wild type phage T4 (Figure 10) and with wild type E. coli and the $p_x$ phage (Figure 11). As shown in these figures, there seems to be a general depression of MR with the elimination of the starvation prior to infection. However, there is no significant difference between those experiments using recombination deficient E. coli or T4 and that found when using wild type bacteria and phage (shown by the dashed line in each figure).

Table 2 summarizes the results of Figures 3-6 and 9-11. The amount of MR was calculated as the ratio of the slope of the multicomplex survival curve to the slope of the monocomplex survival curve. This ratio is termed the MR factor. The number in parentheses is the number of experiments from which the average ratio was calculated.

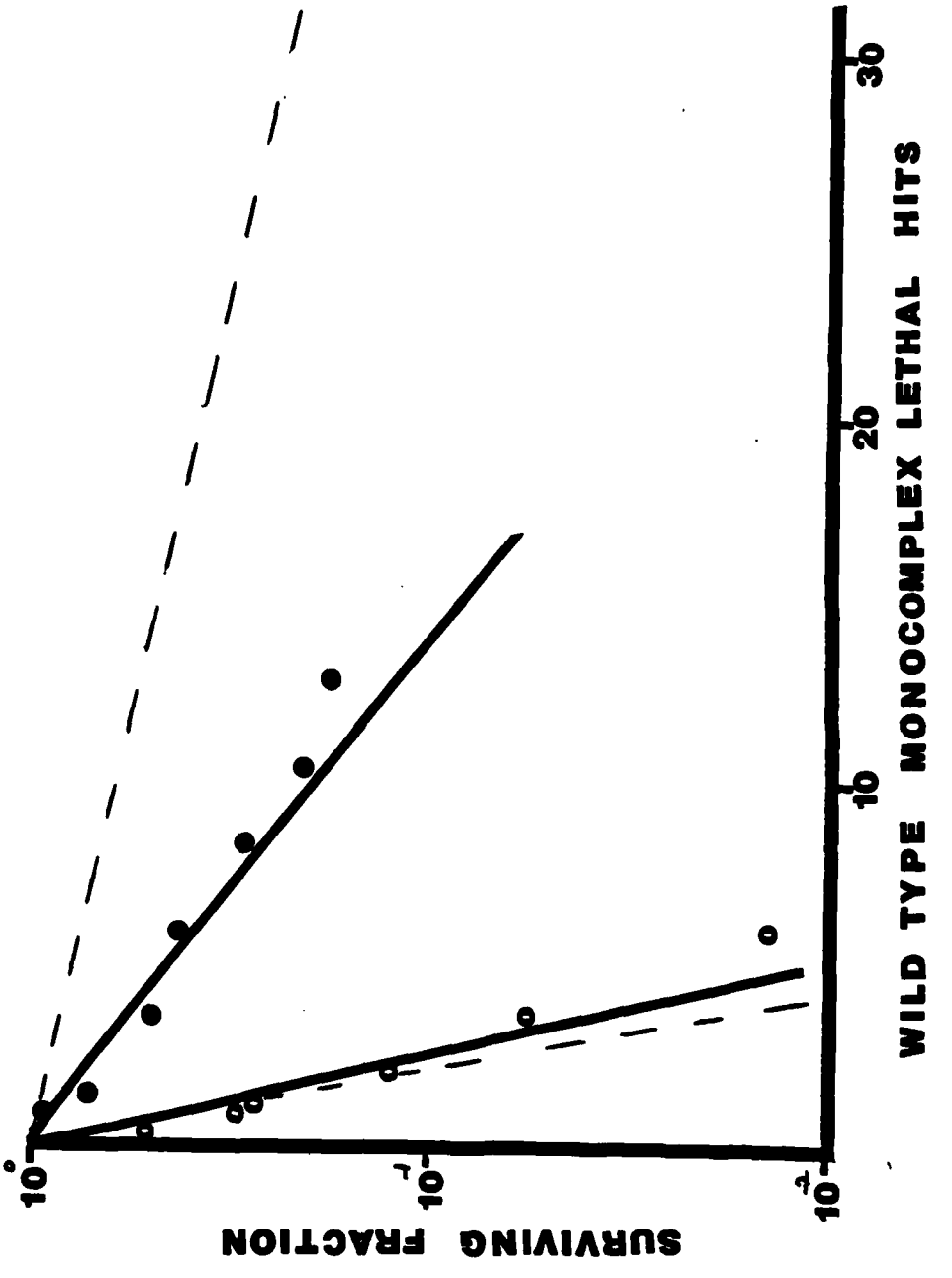


Figure 9. MR with wild type E. coli and T4 without starvation.

●---Multicomplexes
o---Monocomplexes

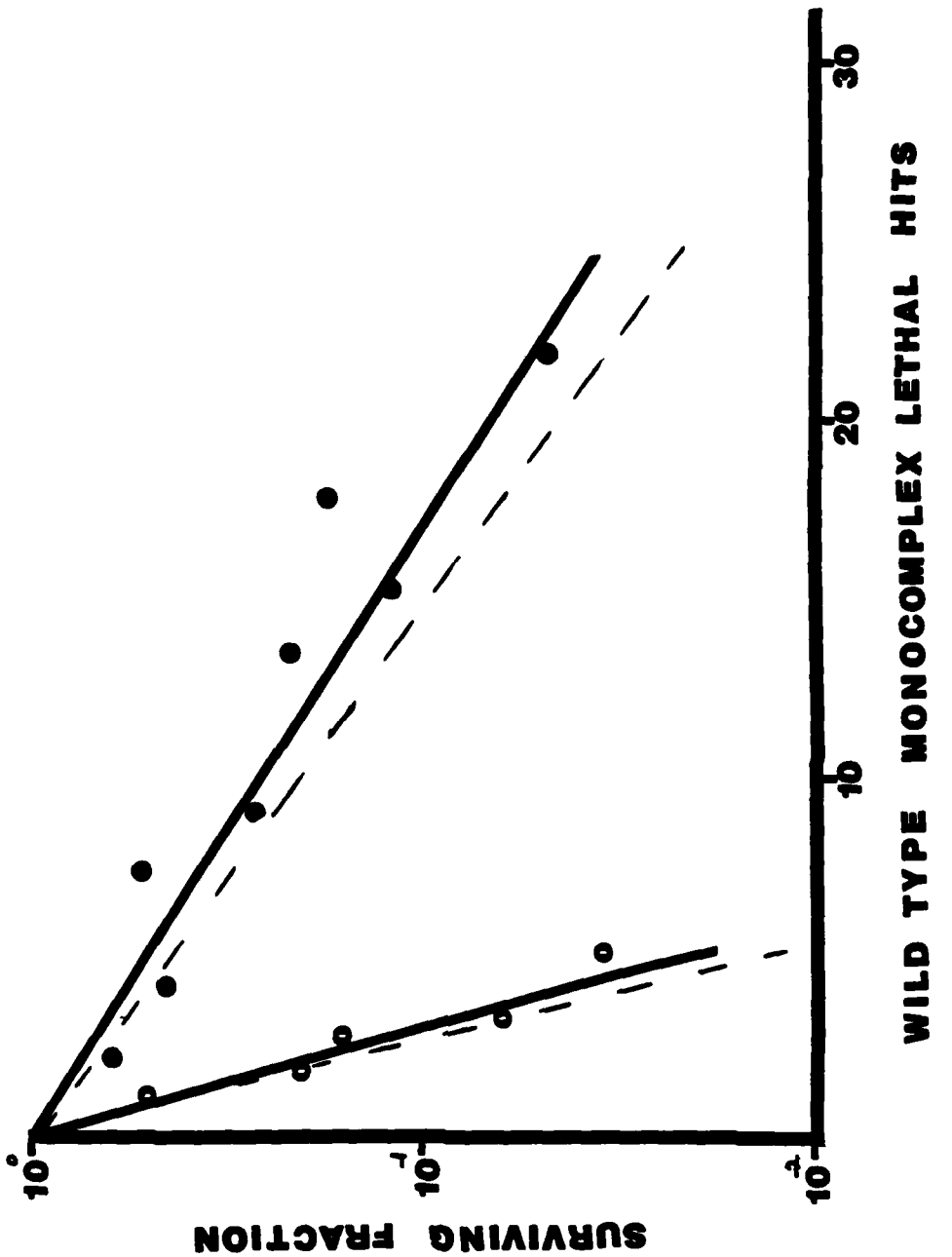


Figure 10. MR with recombination deficient recA⁻ E. coli and wild type T4 without starvation.

●---Multicomplexes
o---Monocomplexes

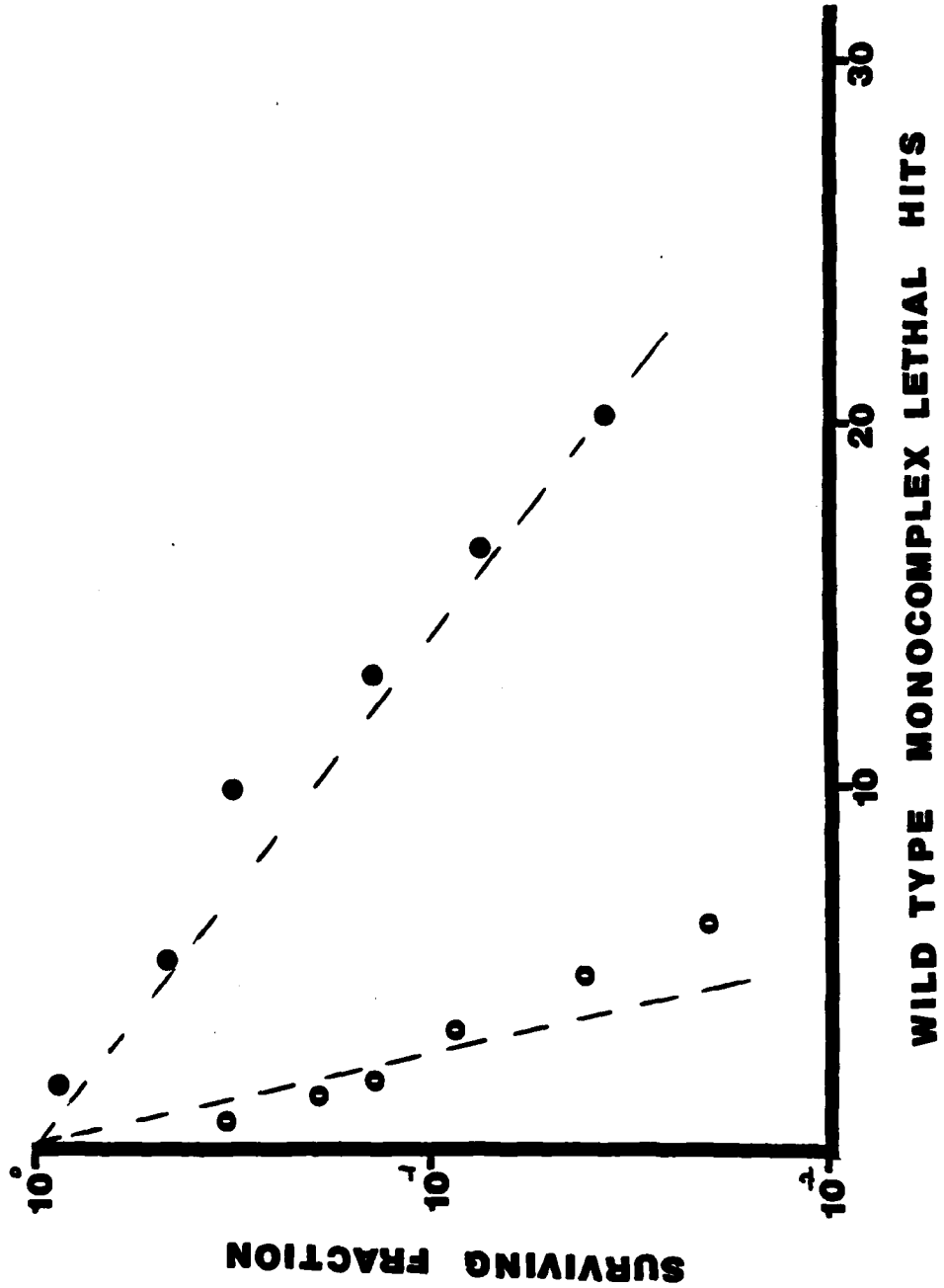


Figure 11. MR with wild type E. coli and recombination deficient uvsX⁻ phage without starvation.

● ---Multicomplexes
o ---Monocomplexes

Table 2. Summary of MR Results.

| Phage | E. coli | Average MR Factor: Starvation | Average MR Factor: No Starvation |
|---|---|---|---|
| T4D (WT) | AT713 (WT) | 11.5 (3) | 4.3 (2) |
| T4D (WT) | GY3428 (recA431) | 3.9 (2) | 4.8 (2) |
| T4D (WT) | GY3448 (recA430) | 4.2 (2) | |
| $p_x$ (uvsX) | AT713 (WT) | 11.3 (2) | 4.4 (2) |

## recA Induction by Starvation

Experiments were performed to test the effect of starvation on the induction of the recA protein of *E. coli* in the absence of phage infection. The results of the recA assay are shown in Figure 12.

The location of the 38,000 molecular weight protein, the recA monomer, is indicated by the arrow. The experiment was conducted using strain AT713 *E. coli* (wild type) with lane 1 taken from cells in log phase growth. Lane 2 depicts the results following UV induction of the SOS pathway (Little and Hanawalt, 1977). Lanes 3 and 4 are from cells after 30 and 60 minutes in starvation conditions respectively. From these results, it was concluded that starvation induced a 38,000MW protein, recA, in a similar manner as UV irradiation.

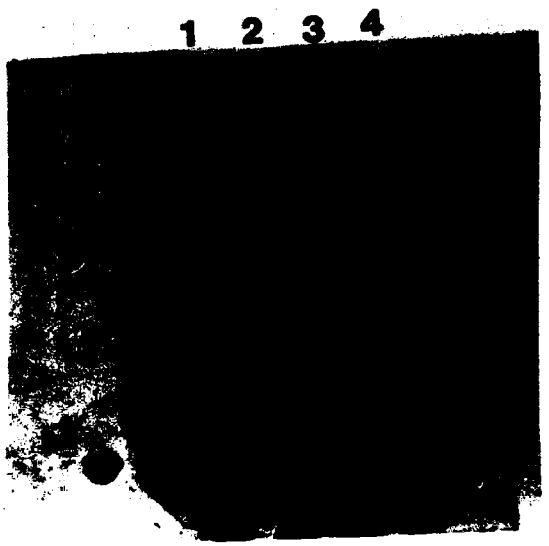


Figure 12. SDS-PAGE of recA assay.
➧ ---Location of 38,000 molecular weight protein (recA) Lane 1 is from log phase cells. Lane 2 is from UV irradiated cells. Lane 3 is from cells starved 30 minutes. Lane 4 is from cells starved for 60 minutes.

## DISCUSSION

The requirement for a fully functional recA gene product in the MR pathway is shown by the decreased MR when a recA mutant is used as the host (Figures 4 & 5) as compared to when a wild type host is used (Figure 3). When recA mutants were utilized that were either deficient in the recombination function (Figure 4) or the protease function (Figure 5) of the enzyme, a reduction in MR of MMC damaged phage of about 66% was observed. This result is consistent with those of Primer and Chan (1978).

To attempt to determine which of the two main functional parts of the recA gene product was involved in the MR pathway, two E. coli strains, described by Morand et al. (1977), were used, each carrying a different recA allele. Strain GY3428 was derived from strain AT713 and carried the recA431 allele. This allele is characterized as being protease proficient, as measured by its ability to induce λ prophage, but recombination deficient. The decrease in MR seen in Figure 4, indicates that the key recombinational events catalyzed by the recA protein, homologous pairing, strand insertion, and branch migration, are required in the MR pathway and that recA is the primary enzyme providing these functions.

The strain GY3448, also derived from AT713 but carrying the recA430 allele, was used to try to determine if the protease function of recA was needed for MR. This allele is deficient in its ability to induce the λ prophage, but retains near normal recombinational capability. Studies by Roberts and Roberts (1981) and Devoret et al. (1983) indicate that recA430 is a protease deficient allele of the recA gene that reduces the UV-induced levels of recA approximately 70%. From the results depicted in Figure 5, it is concluded that constitutive levels of the recA protein and/or some other enzyme under the recA-lexA regulation, is not sufficient to support normal levels of MR. There are three possible reasons for this. First, the constitutive level of the recA protein may not be enough to satisfy the stoichoimetry of the recombinational reactions catalyzed by recA. Second, there are two genetically distinct pathways in the recA controlled SOS response of E. coli, the recBC and the recF pathways. Of these, the recF pathway is reported to be enhanced by increased levels of recA. This pathway is thought to be the one responsible for the integration of ssDNA into the bacterial chromosome. It is possible that recF or some enzyme in its pathway may also be required for MR in increased levels. (Clark, 1973; Lloyd et al., 1983; Lloyd and Thomas, 1983; Smith, 1983)

Third, a T4 substrate for the activated recA protease may exist. The results reported here do not differentiate between these possibilities.

The results depicted in Figures 3 and 6 show there is no difference in MMC treated uvsX mutant phage and identically treated wild type phage. From this it is clear that the uvsX gene product is not required for the survival of MMC treated multicomplexes in either starved or non-starved cells. These results are in agreement with those of Holmes et al. (1980). Also consistent with this finding is the lack of a "shoulder" in the MR curve of MMC treated phage as opposed to that reported in the survival curves of UV treated phage. This shoulder portion of the MR pathway has been shown to be dependent on the uvsX gene (Bernstein, 1981; Bernstein and Wallace, 1983).

Starving the host cells prior to phage infection was initially introduced into the experimental protocol to arrest DNA replication of the infecting phage before adding the MMC so that DNA lesions would be introduced prior to replication. In doing this, it was hoped to eliminate the Luria-Laterjet effect (Luria and Laterjet, 1947) which is a decrease in sensitivity to DNA damaging agents that occurs during intracellular phage replication. When a conspicuous increase in slope of the monocomplex inactivation curve was seen at higher doses of MMC, one possibility was that the phage were succeeding in replicating in some of the cells

prior to the addition of the MMC thus creating, in effect, a multicomplex, i.e. the Luria-Laterjet effect. Treatments 1-3 depicted in Figure 8 were designed to suppress possible DNA replication or to introduce the MMC at such a time that less replication could precede its addition in an attempt to diminish the shift in slope of the monocomplex curves. As indicated, none of the revised treatments altered the shape of this portion of the curve. It was only when the protocol was modified to eliminate the starvation step did the monocomplex inactivation curve reflect only one population of cells rather than the two previously seen. These results were interpreted as implying the existence of a DNA repair mechanism that was induced in response to starvation condtitions.

The effect of starvation on MR was then examined and the results obtained are shown in figures 9-11. In general, the effect of not starving the cells prior to adding the phage resulted in a reduction of the MR comparable to that previously observed in the recA mutant hosts. It is interesting to note that this MR level of approximately 4.5--5.0 is not further reduced when the experiments are conducted using a recombinationally deficient mutant phage or host (Figures 10 and 11). This implies that some of the MR that does occur when there is no starvation may be independent of the recA function.

The results shown in Figure 7 suggest that starvation of E. coli induces the same level of recA mediated DNA repair as the standard UV induction technique. This implies that induction of recA and the resulting stimulation of recombinational repair may have evolved as an adaptive response to a poor, though common, naturally occurring physiological state of the cell.

## Summary

The effect of two recA alleles on the multiplicity reactivation pathway of mitomycin C damaged bacteriophage T4 was studied. It was shown that both primary functions attributed to the enzyme, that of catalyzing homologous pairing and strand transfer reactions of joint molecule formation and that of a highly specific protease, are required for full function of the pathway. Additionally, the T4 gene uvsX, reported to code for a recA like protein was not found to affect the efficiency of MR.

In the course of the investigation, it was noted that starving the host cells prior to phage infection appeared to induce MR. Evidence is presented that this induction occurs by a recA mediated mechanism.

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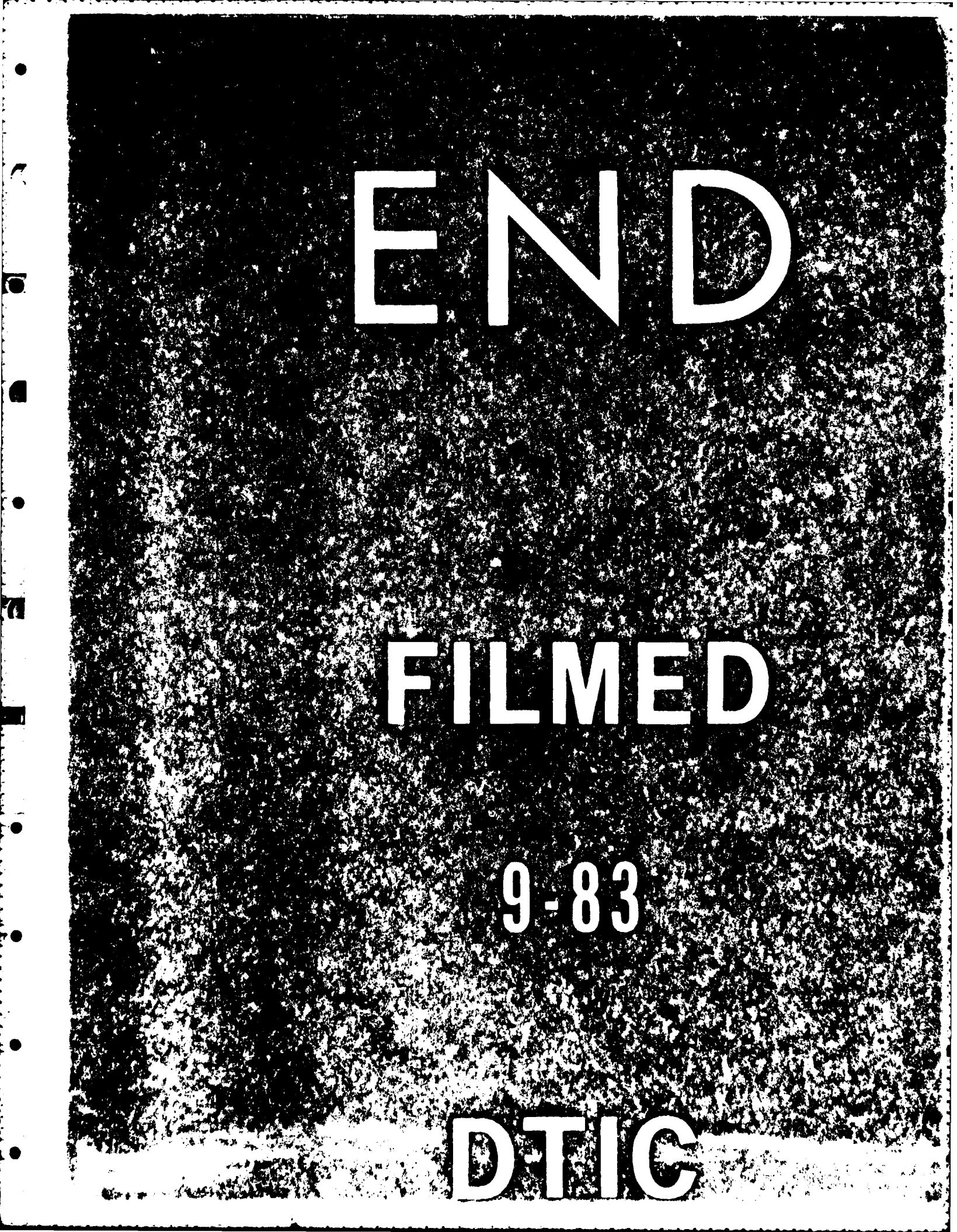
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